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(57) Abstract

A method of producing recombinant transforming growth factor β-induced H3 protein and the use of this protein to accelerate wound healing. H3 promoted adhesion of human dermal fibroblasts to tissue culture plastic. The protein is applied directly to a wound or is used to promote adhesion and spreading of dermal fibroblasts to a solid support such as a nylon mesh which is then applied to the wound. In addition, CHO cells expressing H3 inhibited tumor cell growth.

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FACTOR TO GROW TISSUE EX VIVO

FIELD OF THE INVENTION

The present invention relates to cellular proteins which regulate cell adhesion. In particular, the invention relates to a cellular protein induced by Transforming Growth Factor-β_1 (TGF-β_1) which promotes the adhesion of human dermal fibroblasts and inhibits the adhesion of a number of transformed human cell lines including lung fibroblasts and HeLa cells. The invention also relates to the production of recombinant H3 in Chinese hamster ovary cells and the inhibition of tumor growth in mice injected with these cells.

BACKGROUND OF THE INVENTION

Cell adhesion is involved in a number of critical cellular processes including anchorage to the extracellular matrix and to other cells, growth, differentiation and migration. Cell adhesion is mediated by dimeric transmembrane receptor proteins called integrins (Ruoslahti, (1991) *J. Clin. Invest.*, **87**:1-5; Hynes, (1992) *Cell*, **69**:11-25). Integrins promote cell adhesion to the extracellular matrix, a filamentous network of proteins secreted by cells, by binding to target sequences present in these proteins. The major target sequence recognized by integrins is an arginine-glycine-aspartate (RGD) motif present in numerous substrate proteins including fibronectin, vitronectin and laminin (Hemler, (1990) *Annu. Rev. Immunol.*, **8**:365-400). First identified in fibronectin, RGD has since been shown to be the cellular recognition sequence in many matrix proteins. Other sequence motifs have also been found to promote cell adhesion, including KQAGD found in fibrinogen and PDSGR found in laminin.

TGF-β encompasses a family of dimeric proteins including TGF-β1, TGF-β2, TGF-β3, TGF-β4, and TGF-β5 which regulate the growth and differentiation of many cell types (Barnard et al., (1990) *Biochim. Biophys. Acta.*, **1032**:79-87). Other members of this family include the more distantly related Mullerian inhibitory substance (Cate et al., (1986) Cell, **45**:685-698) and the inhibins (Mason et al., (1985) *Nature*, **318**:659-663).

TGF exhibits a diverse range of biological effects, stimulating the growth of some cell types (Noda et al., (1989) *Endocrinology*, 124:2991-2995) and inhibiting the growth of other cell types (Goey et al., (1989) *J. Immunol.*, 143:877-880; Pietenpol et al., (1990) *Proc. Natl. Acad. Sci. USA*, 87:3758-3762). In regard to cell adhesion, TGF-β increases the expression of collagen and fibronectin (Ignotz et al., (1986) *J. Biol. Chem.*, 261:4337-4345) and accelerates the healing of incisional wounds (Mustoe et al., (1987) *Science*, 237:1333-1335).

Skonier et al. (*DNA Cell Biol.*, 11:511-522, 1992) cloned and sequenced a TGF-β1-induced gene isolated from a human lung adenocarcinoma cell line by constructing cDNA libraries from both TGF-β1-stimulated and unstimulated cells and screening the libraries by subtractive hybridization. This gene encoded a 683 amino acid protein called βIG-H3 (H3) which contained a carboxy-terminal RGD sequence. The protein also contained four internal repeats with limited homology to *Drosophila* fasciclin I, an extrinsic membrane protein thought to be involved in growth cone guidance, and a PDSAK sequence similar to the PDSGR active binding domain of laminin. The presence of these sequence motifs indicated that H3 could be involved in cell adhesion.

Numerous attempts have been made at increasing fibroblast adhesion to substrates. The main approach has involved the use of RGD-containing peptides (Quaglino, Jr., et al., (1991) *J. Invest. Dermatol.*, 97:34-42; *Peptide Res.*, 5:331-335; Agrez et al., (1991) *Cell Regul.*, 2:1035-1044), although this method has had limited success. U.S. patent 4,963,489 to Naughton et al. discloses a three-dimensional matrix and its use as a framework for a multi-layer cell culture system for the production of a number of cells and/or tissues by culturing desired cell types on a stromal cell layer.

There are currently no simple, effective methods for stimulating cell spreading and adhesion at wound sites to promote rapid wound healing. Thus, there is a need for substances able to promote attachment and spreading of cells,

particularly fibroblasts, to facilitate this important process. Such a substance and its use in wound healing and tissue engineering applications are described herein.

SUMMARY OF THE INVENTION

One embodiment of the present invention is a method for enhancing the attachment of cells to a solid support by coating the support with an effective cell attachment-enhancing amount of H3 protein prior to contacting the support with the cells. Preferably, the cells are mammalian; most preferably, the cells are human. Further, the cells may be either fibroblasts, epithelial cells or keratinocytes. Advantageously, the H3 is either recombinant or derived from human fibroblasts. According to another aspect of this preferred embodiment, the solid support is a three dimensional scaffold which may be in the form of a sheet or mesh. Suitable materials for the solid support include polytetrafluoroethylene, polystyrene, polypropylene, polyacrylates, polyvinyl compounds, polycarbonate, nitrocellulose, cellulose, polyglycolic acid, catgut sutures and gelatin.

The invention also provides an article of manufacture comprising a solid support coated with H3. Preferably, the solid support is a three-dimensional scaffold which may be either a sheet or mesh. Suitable materials for the solid support include polytetrafluoroethylene, polystyrene, polypropylene, polyacrylates, polyvinyl compounds, polycarbonate, nitrocellulose, cellulose, polyglycolic acid catgut sutures and gelatin. Advantageously, the H3 is either recombinant or derived from human fibroblasts.

Another embodiment of the invention is a method for inhibiting tumor growth comprising contacting the tumor with a DNA construct operably encoding H3. This method may further comprise radiation and chemotherapy treatment.

The invention further provides a method for accelerating wound healing by contacting the wound with an effective healing-promoting amount of H3. Preferably, the H3 is contained in a topical pharmaceutical formulation consisting

of an aqueous solution, gel, cream, paste, lotion, spray, suspension, dispersion, salve or ointment. In accordance with another aspect of this embodiment, the H3 is either recombinant or derived from human fibroblasts. Preferably, the wound is either a skin ulcer, burn, laceration or surgical incision.

Still another embodiment of the invention is a shaped article comprising a solid support, H3 protein coated onto the support and cells adhering to the H3-coated solid support. Preferably, the solid support is a three dimensional scaffold which may be either a sheet or mesh. Preferably, the sheet is made of either polytetrafluoroethylene, polystyrene, polypropylene, polyacrylates, polyvinyl compounds, polycarbonate, nitrocellulose, cellulose, polyglycolic acid, catgut sutures or gelatin. According to another aspect of this embodiment, the cells are either fibroblasts, epithelial cells or keratinocytes.

The invention also provides a method for accelerating wound healing comprising applying the solid support mentioned hereinabove to the wound.

According to another aspect of the invention, there is provided a pharmaceutical composition comprising H3 in a pharmaceutically acceptable carrier. Preferably, the carrier is either an aqueous solution, gel, cream, paste, lotion, spray, suspension, salve or ointment.

Yet another embodiment of the invention is a method for accelerating wound healing comprising contacting the wound at least daily with between about 10 μg and about 10 mg H3.

Further, the invention provides a method for accelerating wound healing comprising contacting the wound with a shaped article, the shaped article comprising a solid support coated with between about 0.1 $\mu g/mm^2$ and about 10 $\mu g/mm^2$ H3 protein and fibroblasts adhering to the H3 protein.

According to another aspect of the invention, there is provided a method of producing recombinant H3 protein comprising the following steps:

inserting the DNA sequence encoding H3 into an expression vector containing a selectable marker, the H3 DNA sequence in operable juxtaposition to a heterologous promoter;

transfecting chinese hamster ovary cells with the expression vector;

culturing the cells in a selection medium ;

selecting and expanding positive clones; and

purifying the H3 protein.

Preferably, the promoter is the cytomegalovirus promoter and the expression vector is pEE-14.

Brief Description of the Drawings

Figure 1 shows the growth curve for H3-producing CHO cell clones. Control CHO/pEE-14, CHO/H3cl.A13 (A-13) and CHO/H3cl.A2 (A-2) cells were seeded at 5 x 10^5 cells in 100 mm dishes, grown for the indicated times, trypsinized and counted. Data points represent the average of duplicate samples. The x-axis shows the cell growth time and the y-axis shows the cell number.

Figure 2 shows the H3-mediated inhibition of A549 cell adhesion. Tissue culture wells were coated with either varying amounts of purified recombinant H3 protein or control pEE-14 media and 5 x 10^5 A549 cells were incubated in the wells for two hours at 37°C. Wells were washed with PBS and the remaining attached cells were counted. The x-axis shows the amount of H3 used (μg) and the y-axis shows the number of attached A549 cells.

Figure 3 is a graph showing the increase in human dermal fibroblast adherence with increasing concentrations of recombinant H3 after a 2.5 hour cell attachment period. The concentration of added H3 is shown on the x-axis and the cell adherence as reflected by the absorbance at 405 nm is shown on the y-axis.

Figure 4 is a graph illustrating the increase in fibroblast adherence to recombinant H3-coated microtiter wells over time. The adhesion time is shown on the x-axis and the absorbance at 405 nm is shown on the y-axis.

Detailed Description of the Invention

The present invention discloses the stimulatory and inhibitory action of the TGF-β1-induced H3 protein on cell adhesion. In addition, the production of recombinant H3 in CHO cells and the H3-mediated inhibition of tumor formation is also disclosed.

Recombinant H3 protein was produced in Chinese hamster ovary (CHO) cells by transfecting a DNA construct containing the cDNA encoding H3 operably linked to a cytomegalovirus (CMV) promoter. A number of promoters well known in the art are also contemplated including the Simian Virus 40 (SV40), Rous sarcoma virus (RSV), adenovirus and any other promoter capable of being operably linked to H3 such that production of recombinant protein occurs in transfected mammalian cells. The construct also preferably contains a eukaryotic selectable marker encoding drug resistance to allow identification of positive transfectants. Nonlimiting examples of such selectable markers include methionine sulfoxide (MSX), dihydrofolate reductase, hygromycin and neomycin. Transfection may be accomplished by a number of techniques well known in the art, including but not limited to calcium phosphate precipitation, lipofection, electroporation, and DEAE-dextran mediated delivery. The transfected cells are then cultured in a medium containing a toxic substance to allow selection of transfectants expressing the drug resistance gene. These positive clones are pooled and expanded using conventional tissue culture techniques.

Since the H3 protein is secreted, it accumulates in the conditioned medium of the H3 CHO transfectants. The protein may then be isolated by conventional protein purification techniques well known in the art. The preferred method of isolation involves ammonium sulfate precipitation and gel filtration column chromatography, although any other purification method including, but not limited to, affinity chromatography, ion exchange chromatography, adsorption chromatography and high performance liquid chromatography is

also contemplated.

H3 protein is also produced by human foreskin fibroblasts, both in their unstimulated and TGF β1-stimulated states; however, the level of H3 produced is increased in TGF β1-stimulated cells. The protein is secreted by the fibroblasts and accumulates in the culture medium.

Since the H3 protein both inhibited and promoted cell adhesion, it has applications in cancer therapy and wound healing. H3 inhibited the adhesion of the A549, HeLa and WI-38 transformed cell lines, indicating its utility as an inhibitor of cancer cell adhesion to both other cells and to the extracellular matrix. Importantly, CHO cells transfected with the H3 cDNA were severely compromised in their ability to form tumors in immunosuppressed mice compared to control cells transfected with the vector alone. Thus, H3 will have utility in gene therapy. Tumor cells transfected *in vivo* with a DNA construct encoding H3 operably linked to a heterologous promoter will be growth inhibited. Gene therapy using H3 may be combined with conventional chemotherapy and radiation treatment to increase the overall treatment efficacy. Methods of introduction of the DNA construct to tumor cells include direct injection and intravenous administration of an antibody-DNA conjugate in which the antibody has affinity for a tumor cell antigen and is internalized by the tumor cell.

H3 was also determined to specifically promote the adhesion of human dermal fibroblasts to tissue culture plates. Thus, H3 will have utility in promoting wound healing, a process in which increased fibroblast adhesion and spreading is desired. Such wounds include burns, skin ulcers, lacerations, surgical incisions and the like. The recombinant H3 protein may be directly applied to the wound in a sterile physiological solution such as saline in an effective fibroblast adhesion-accelerating amount. This is a simple method which does not require growing fibroblasts prior to application to the wound and obviates the need to obtain an individual's own cells for treatment.

Alternatively, the H3 may be incorporated into a pharmaceutical composition. Nonlimiting examples of particularly preferred compositions of H3 for topical administration include lotions, creams, gels, salves, sprays, dispersions, suspensions, pastes and ointments. The preparations may further advantageously include preservatives, antioxidants, antibacterials, antifungals, antiosmotic agents and similar materials in composition and quantity as is conventional. For assistance in formulating the compositions of the present invention, one may refer to Remington's Pharmaceutical Sciences, 15th Ed., Mack Publishing Co., Easton, PA (1975).

Although the amount applied and frequency of application will vary depending on the severity and size of the wound, contemplated amounts of H3 range from about 10 μg to about 10 mg per application. The composition may be applied daily, every other day or every several days.

In another embodiment, a shaped article such as a three dimensional scaffold, fabric, sheet, mesh or other appropriate article may be coated with H3. Although the amount of H3 used to coat the article will vary depending on the size and composition of the article, it is estimated that between about 0.1 μg and about 100 μg per mm^2 will be sufficient. The article may be coated by spraying with or immersion in a solution containing H3 in a concentration ranging from about 10 μg/ml to about 10 mg/ml. In a preferred embodiment, the concentration of H3 is between about 50 μg/ml and about 1 mg/ml. Other coating methods are also within the scope of the invention. The optimum coating amount of H3 for promoting cell attachment to a solid support may easily be determined by one of ordinary skill in the art.

The article may be constructed of a number of inert, biocompatible, nontoxic materials including nylon, polyester, polytetrafluoroethylene (PTFE), polystyrene, polypropylene, polyacrylates, polyvinyl compounds, polycarbonate, nitrocellulose, cellulose, polyglycolic acid (PGA), catgut sutures, gelatin, or any material to which H3 can be applied

and to which fibroblasts will adhere. Any of these materials may be woven into a mesh to form a three-dimensional matrix. One suitable nylon mesh for use in the present invention is NITEX™, a nylon filtration mesh having an average pore size of 210 μm and an average nylon fiber diameter of 90 μm (Tetko, Inc., New York).

Cells are then cultured on the H3-coated article under standard growth conditions including nutrients, antibiotics and growth factors, resulting in a three dimensional array of cells. Although human cells are preferred, cells from other mammals including, but not limited to, monkeys, mice and rabbits are also contemplated. Other cell types suitable for application to the H3-coated article include keratinocytes, epithelial cells, epidermal cells and any desired cell type capable of adhering to the H3-coated article. One having ordinary skill in the art of cell biology could easily determine whether a given cell type was capable of binding to an H3-coated article.

Although cells may be obtained from a number of sources including fetal and adult tissues, they are preferably isolated from the same individual who is to later receive the transplanted/implanted cells and/or tissues grown according to the present invention so as to reduce the chance of immunological rejection. The resulting three dimensional array of cells is structurally analogous to *in vivo* tissue.

The three dimensional culture may be transplanted or implanted into a patient, seeding the damaged area and providing a nucleus for subsequent cell attachment, resulting in accelerated closure of the wounded area. This will promote healing by stimulating proliferation of cells in the surrounding tissue. Transplanted fibroblast cultures on wound sites will decrease the chances of bacterial infection by serving as a barrier, similar to normal skin. The three dimensional culture may be used as either a permanent treatment or may serve as a temporary barrier to infection and fluid loss until a skin graft can be performed. Where the three-dimensional culture is to be implanted *in vivo*, it

may be preferable to use biodegradable matrices such as PGA, catgut suture material or gelatin, for example.

To obtain sufficient amounts of recombinant H3 protein for *in vitro* analysis, the cDNA was expressed in mammalian cells as described below.

Example 1

Production of Recombinant H3

The glutamine synthetase expression system (Celltech, Berkshire, United Kingdom) was used to express H3 in recombinant chinese hamster ovary (CHO) cells (Cockett et al., (1990) *Biotechnology*, 8:662-667; Bebbington et al., (1992) *Biotechnology*, 10:169-175). The H3 coding region (Skonier et al., (1992) *DNA Cell Biol.*, 11:511-522; SEQ ID NO: 1) was cloned into the expression vector pEE-14 which contains a cytomegalovirus (CMV) promoter (Celltech) and transfected into CHO cells using calcium phosphate precipitation as instructed by the manufacturer. Transfectants were selected using 25 μM methionine sulfoxide (MSX) and individual clones were selected and expanded. Clones secreting H3 were identified by immunoblotting of conditioned serum-free medium with a polyclonal antibody to H3. Positive clones were designated CHO/H3cl.A13, CHO/H3cl.A19 and CHO/H3cl.A2g. Control CHO cells transfected with empty vector are designated as control CHO/pEE-14 cells.

When immunoblotting was performed after SDS-PAGE analysis under non-reducing conditions, CHO/H3cl.A13 cells secreted a protein migrating on SDS gels at about 68 kDa. This protein was not secreted in cells transformed with the vector alone. The protein was absent in control CHO cells. When immunoblotting was performed after SDS-PAGE under reducing conditions, three closely spaced bands were observed which were absent in control CHO cells. Since there are no predicted sites of N-linked glycosylation in the deduced βIG-H3 protein sequence (Skonier et al., 1992), and since neuraminidase treatment did not affect electrophoretic mobility, the observed heterogeneity may be due to carboxy-terminal processing, sulfation or methylation.

The secreted recombinant H3 protein was purified and sequenced as described below.

Example 2

Purification of recombinant H3

Serum-free conditioned medium from CHO/H3cl.A13 cells was precipitated with 50% ammonium sulfate at 4°C for 20 hours and centrifuged for 30 minutes at 30,000 x g. The pellet was dissolved in phosphate buffered saline (PBS) and applied to a BioSil TSK-250 gel filtration column (BioRad, Richmond, CA) equilibrated with PBS. Fractions containing H3 were identified by immunoblotting, pooled, aliquoted and stored at -70°C.

Proteins were fractionated by SDS-PAGE and transferred to a ProBlott membrane (Applied Biosystems, Foster City, CA) using a mini-transblot electrophoretic transfer cell (BioRad) as previously described (Matsudaira, (1987) *J. Biol. Chem.*, 262:10035-10038). The membrane was stained with Coomassie Brilliant Blue, destained and the 68 kDa band was excised for amino-terminal sequence analysis.

Samples were sequenced in a pulsed-liquid phase protein sequencer (Applied Biosystems model 476A) equipped with a vertical cross-flow reaction cartridge. The phenylthiohydantoin (pth) amino acid derivatives were analyzed by reversed-phase high performance liquid chromatography (HPLC). Data reduction and quantitation were performed on a Macintosh IIsi computer (Apple Computers, Inc.) and model 610A chromatogram analysis software (Applied Biosystems).

Since the growth rate of the CHO/H3cl.A13 and CHO/H3cl.A2g clones was slower than that of the control cells (Figure 1) and since the two clones reached an overall lower saturation density, the tumorigenicity of the cells was assessed as described below.

Example 3

Reduced tumorigenicity of CHO cells expressing H3

Three independently selected H3-expressing clones were injected (3 x 10^7 cells per injection) subcutaneously into

the backs of female athymic nude mice (Harlan Sprague Dawley, Indianapolis, IN) and tumors were evaluated at 4 weeks. As shown in Table 1, while the control cells readily formed tumors, CHO cells expressing H3 were significantly impaired in their ability to form tumors. The single tumor arising from the CHO/H3cl.A13 cells remained small (3 x 3 mm) over 10 weeks of observation, while the control cells typically produced a tumor measuring 15 x 20 mm by 4 weeks.

Table 1

Clone	# tumors/# animals injected
Control CHO/pEE-14	8/10
CHO/H3cl.A19	0/10
Control CHO/pEE-14	8/10
CHO/H3cl.A13	1/10
Control CHO/pEE-14	7/10
CHO/H3cl.A2g	0/10

Since H3 is a secreted protein with four regions of internal homology to fasciclin I and contains an RGD motif common in proteins modulating cell attachment, an adhesion assay using various human cell lines was performed as described in the following example.

Example 4

Inhibition of cell adhesion by H3

A549 human lung adenocarcinoma (American Type Culture Collection (ATCC), Rockville, MD, ATCC CCL 185), HeLa (ATCC CCL 2), WI-38 human lung fibroblasts (ATCC CCL 75) and CHO cells (ATCC CRL 9096) were grown in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal calf serum (FCS).

Cell adhesion assays similar to those used to identify proteins and their active domains involved in cell attachment were carried out on H3 attached to Costar 6-well plates (Graf et al., (1987) *Cell*, 48:989-996; Dustin et al., (1989) *Nature*, 341:619-624). Individual wells of a 24-well tissue culture dish were incubated for 2 hours at 22°C with 7.5 μg purified recombinant H3, BSA or serum-free medium from

control CHO/pEE-14 cells purified in a similar fashion to H3. A549 cells (2 x 10^5 cells/well) were added in serum-free medium and allowed to attach for 2 hours at 37°C.. Cells were removed and the wells were washed twice with PBS and photographed.

The results show that in the presence of H3, the cells did not attach to the wells. Conversely, attachment occurred after plating on equivalent amounts of BSA or control CHO/pEE-14 protein. This effect was concentration-dependent (Figure 2). When A549 cells were plated in the presence of 7.5 μg H3, only about 200 cells remained attached to the plate; with 1.875 μg protein, about 2,500 cells remained attached. In contrast, when A549 cells were plated in the presence of 7.5 μg BSA, about 15,000 cells remained attached to the plate. Similar results were obtained with HeLa, WI-38 and CHO cells.

Surprisingly, carboxy-terminal sequencing of the recombinant H3 protein revealed that the RGD sequence was not present, most likely due to carboxy-terminal processing of H3. Therefore, it is possible that the anti-adhesion activity of H3 is not mediated through the RGD sequence. Hence, another sequence motif such as the PDSGR laminin adhesion domain or an as yet unidentified sequence motif may be responsible for the anti-adhesion activity mediated by H3.

Example 5

Promotion of fibroblast adhesion by H3

Human foreskin fibroblasts isolated at Advanced Tissue Sciences, La Jolla, CA, were grown in monolayer culture. This cell type is also available from American Type Culture Collection, Rockville, MD (ATCC CRL 1634 and CRL 1635). Two hours before the experiment, cells were included in all subsequent washing and adhesion solutions. Cells were harvested by incubation with 0.25% trypsin in PBS lacking calcium and magnesium salts. Cells were washed twice with Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS), twice with DMEM only, counted and resuspended in DMEM containing 10% heat treated bovine serum

albumin (BSA).

Recombinant H3 was purified as described (Skonier et al, 1992). Recombinant H3 and human serum were diluted in water to a final volume of 100 ul and allowed to air-dry in the microtiter wells overnight. It is well known that the predominant cell adhesion protein present in serum is fibronectin. The proteins were rehydrated in 200 μl PBS for 15 minutes, removed and non-specific binding sites blocked with 1% BSA in PBS for 3 hours at room temperature. The PBS was removed and the wells were washed twice with 200 μl PBS. Approximately 30,000 human foreskin fibroblasts in 100 μl DMEM were added to each well and allowed to attach to the substrates at 37°C. At the appropriate times, cell attachment was quantitated by measuring absorbance at 405 nm of a p-nitrophenol derivatized chromogenic substrate for hexosaminidase as described by Landegren (*J. Immunol. Methods*, 67:379-388, 1984).

The results indicated that H3 promoted the attachment of human dermal fibroblasts in a concentration-dependent manner (Figure 3). Maximum adhesion occurred at about 30 μg H3/well and remained fairly stable up to 50 μg H3/well. The optimum time for adhesion on the H3 substrate was approximately 2.5 hours (Figure 4). Few cells plated on PBS-BSA adhered to the wells and those that did appeared rounded. In contrast, cells plated on serum-coated or H3-coated wells adhered very well and appeared elongated and spread out as determined by light microscopy. This indicates that H3 can promote the adhesion of dermal fibroblasts and has implications in tissue engineering to promote attachment and spreading of dermal fibroblasts on two and three dimensional scaffolds.

Example 6

Production of H3 by human fibroblasts

Primary human foreskin fibroblasts (PHFF) were cultured in DMEM containing 10% FBS, 2 mM L-glutamine and minimal essential amino acids. Recombinant TGF-β was prepared as described (Gentry et al., *Mol. Cell Biol.*, 8:4162-4168) and used at 20 ng/ml).

Cells were metabolically labeled with 200 μCi/ml [^{35}S]-translabel (ICN, Irvine, CA) in DMEM containing 5% dialyzed fetal calf serum for four hours, and either stimulated with TGF β or left untreated. Supernatants were immunoprecipitated with either anti-H3 antiserum or normal rabbit serum. Immunoprecipitates were analyzed by SDS-PAGE. Gels were dried and exposed to Fuji x-ray film (Sigma, St. Louis, MO). In both TGF β-treated and untreated cells, the anti-H3 antiserum immunoprecipitated a protein having a molecular weight of about 70 kDa. This protein was unreactive with normal rabbit serum. The amount of protein secreted into the culture medium by TGF β-stimulated cells was increased about three fold compared to unstimulated cells.

Example 7

Acceleration of wound healing by H3

Patients having skin ulcers are topically administered 200 μg recombinant H3 prepared according to Example 1 daily in the form of a cream directly to one ulcer. A second ulcer is treated daily with a control cream lacking H3. The healing rate of both ulcers is monitored over time to show the acceleration of wound healing promoted by H3.

A similar experiment is also performed using an H3-coated three dimensional nylon mesh scaffold seeded with human dermal fibroblasts. The scaffold is applied to one ulcer, while a second ulcer is treated with an scaffold coated with only H3 and a third ulcer is treated with an empty scaffold. The improvement of the ulcers is monitored to show the promotion of wound healing by the fibroblast-coated nylon mesh scaffold.

It should be noted that the present invention is not limited to only those embodiments described in the Detailed Description. Any embodiment which retains the spirit of the present invention should be considered to be within its scope. However, the invention is only limited by the scope of the appended claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

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(2) INFORMATION FOR SEQ ID NO:1:

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(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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TACTTCACCA ACTGCAAGCA GTGGTACCAA AGGAAAATCT GTGGCAAATC AACAGTCATC 240

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TACACGGACC GCACGGAGAA GCTGAGGCCT GAGATGGAGG GGCCCGGCAG CTTCACCATC 420

TTCGCCCCTA GCAACGAGGC CTGGGCCTCC TTGCCAGCTG AAGTGCTGGA CTCCCTGGTC 480

AGCAATGTCA ACATTGAGCT GCTCAATGCC CTCGCGTACC ATATGGTGGG CAGGCGAGTC 540

CTGACTGATG AGCTGAAACA CGGCATGACC CTCACCTCTA TGTACCAGAA TTCCAACATC 600

CAGATCCACC ACTATCCTAA TGGGATTGTA ACTGTGAACT GTGCCCGGCT CCTGAAAGCC 660

GACCACCATG CAACCAACGG GGTGGTGCAC CTCATCGATA AGGTCATCTC CACCATCACC 720

AACAACATCC AGCAGATCAT TGAGATCGAG GACACCTTTG AGACCCTTCG GGCTGCTGTG 780

GCTGCATCAG GGCTCAACAC GATGCTTGAA GGTAACGGCC AGTACACGCT TTTGGCCCCG 840

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GGGGACATGC TCACTATCAA CGGGAAGGCG ATCATCTCCA ATAAAGACAT CCTAGCCACC 1080

AACGGGGTGA TCCACTACAT TGATGAGCTA CTCATCCCAG ACTCAGCCAA GACACTATTT 1140

GAATTGGCTG CAGAGTCTGA TGTGTCCACA GCCATTGACC TTTTCAGACA AGCCGGCCTC 1200

GGCAATCATC TCTCTGGAAG TGAGCGGTTG ACCCTCCTGG CTCCCCTGAA TTCTGTATTC 1260

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ACAAATGGCG TGGTCCATGT CATCACCAAT GTTCTGCAGC CTCCAGCCAA CAGACCTCAG 1920

GAAAGAGGGG ATGAACTTGC AGACTCTGCG CTTGAGATCT TCAAACAAGC ATCAGCGTTT 1980

TCCAGGGCTT CCCAGAGGTC TGTGCGACTA GCCCCTGTCT ATCAAAAGTT ATTAGAGAGG 2040

ATGAAGCAT 2049

WHAT IS CLAIMED IS:

1. A method for enhancing the attachment of cells to a solid support comprising coating said support with an effective cell attachment-enhancing amount of H3 protein prior to contacting said support with said cells.

2. The method of Claim 1, wherein said cells are mammalian.

3. The method of Claim 2, wherein said cells are human.

4. The method of Claim 3, wherein said cells are selected from the group consisting of: fibroblasts, epithelial cells and keratinocytes.

5. The method of Claim 1, wherein said H3 is recombinant.

6. The method of Claim 1, wherein said H3 is derived from human fibroblasts.

7. The method of Claim 1, wherein said solid support is a three dimensional scaffold.

8. The method of Claim 7, wherein said solid support comprises a sheet or mesh.

9. The method of Claim 8, wherein said solid support is made of a material selected from the group consisting of: polytetrafluoroethylene, polystyrene, polypropylene, polyacrylates, polyvinyl compounds, polycarbonate, nitrocellulose, cellulose, polyglycolic acid catgut sutures and gelatin.

10. An article of manufacture comprising a solid support coated with H3.

11. The article of Claim 10, wherein said solid support is a three-dimensional scaffold.

12. The article of Claim 11, wherein said solid support comprises a sheet or mesh.

13. The article of Claim 12, wherein said solid support is made of a material selected from the group consisting of: polytetrafluoroethylene, polystyrene, polypropylene, polyacrylates, polyvinyl compounds, polycarbonate, nitrocellulose, cellulose, polyglycolic acid, catgut sutures

and gelatin.

14. The method of Claim 10, wherein said H3 is recombinant.

15. The method of Claim 10, wherein said H3 is derived from human fibroblasts.

16. A method for inhibiting tumor growth comprising contacting said tumor with a DNA construct operably encoding H3.

17. The method of Claim 16, further comprising radiation and chemotherapy treatment.

18. A method for accelerating wound healing comprising contacting said wound with an effective healing-promoting amount of H3.

19. The method of Claim 18, wherein said H3 is contained in a topical pharmaceutical formulation selected from the group consisting of: aqueous solutions, gels, creams, pastes, lotions, sprays, suspensions, dispersions, salves and ointments.

20. The method of Claim 18, wherein said H3 is recombinant.

21. The method of Claim 18, wherein said H3 is derived from human fibroblasts.

22. The method of Claim 18, wherein said wound is selected from the group consisting of: skin ulcers, burns, lacerations and surgical incisions.

23. A shaped article comprising a solid support, H3 protein coated onto said support and cells adhering to said H3-coated solid support.

24. The article of Claim 23, wherein said solid support is a three dimensional scaffold.

25. The article of Claim 24, wherein said solid support comprises a sheet or mesh.

26. The article of Claim 25, wherein said sheet is made of a material selected from the group consisting of: polytetrafluoroethylene, polystyrene, polypropylene, polyacrylates, polyvinyl compounds, polycarbonate, nitrocellulose, cellulose, polyglycolic acid, catgut sutures

and gelatin.

27. The article of Claim 23, wherein said cells are selected from the group consisting of: fibroblasts, epithelial cells and keratinocytes.

28. A method for accelerating wound healing comprising applying the solid support of Claim 25 to said wound.

29. A pharmaceutical composition comprising H3 in a pharmaceutically acceptable carrier.

30. The composition of Claim 29, wherein said carrier is selected from the group consisting of: aqueous solutions, gels, creams, pastes, lotions, sprays, suspensions, salves and ointments.

31. A method for accelerating wound healing comprising contacting said wound at least daily with between about 10 μg and about 10 mg H3.

32. A method for accelerating wound healing comprising contacting said wound with a shaped article, said shaped article comprising a solid support coated with between about 0.1 $\mu g/mm^2$ and about 10 $\mu g/mm^2$ H3 protein and fibroblasts adhering to said H3 protein.

33. A method of producing recombinant H3 protein comprising the following steps:

inserting the DNA sequence encoding H3 into an expression vector containing a selectable marker, said H3 DNA sequence in operable juxtaposition to a heterologous promoter;

transfecting chinese hamster ovary cells with said expression vector;

culturing said cells in a selection medium ;

selecting and expanding positive clones; and

purifying said H3 protein.

34. The method of Claim 33, wherein said promoter is the cytomegalovirus promoter.

35. The method of Claim 33, wherein said expression vector is pEE-14.

36. A DNA construct operably encoding H3 for use in the inhibition of tumor growth.

37. A composition comprising H3 for use in accelerating wound healing.

38. The solid support of Claim 25 for use in accelerating wound healing.

39. A composition comprising between about 10 μg and about 10 mg H3 for use in accelerating wound healing.

40. A shaped article comprising a solid support coated with between about 0.1 $\mu g/mm^2$ and about 10 $\mu g/mm^2$ H3 protein and fibroblasts adhering to said H3 protein for use in accelerating wound healing.

FIG. 1

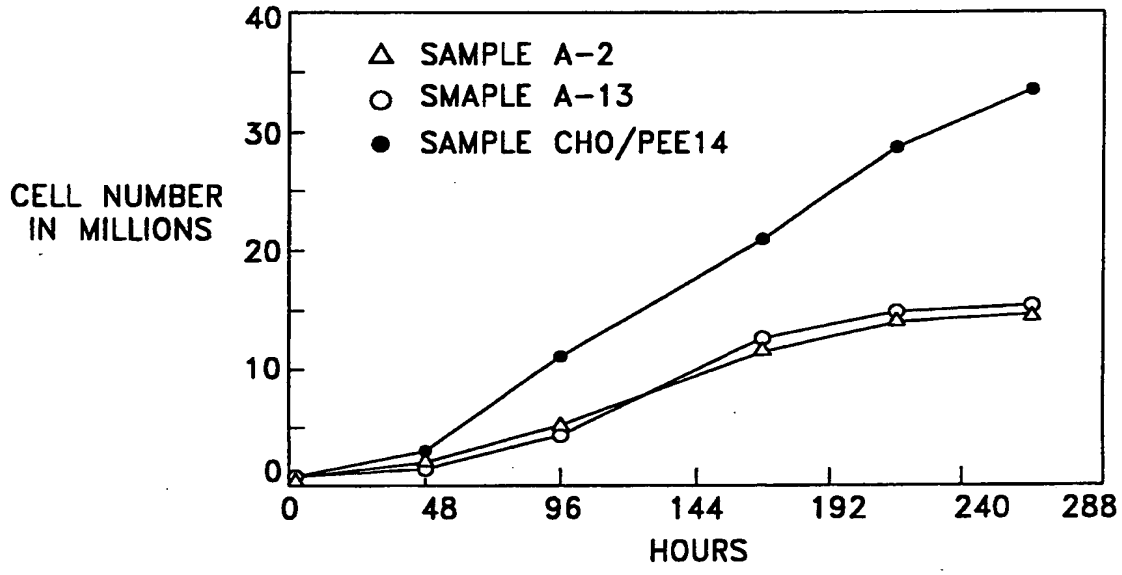

CELL NUMBER IN MILLIONS
40
30
20
10
0
△ SAMPLE A-2
○ SMAPLE A-13
● SAMPLE CHO/PEE14
0
48
96
144
192
240
288
HOURS

FIG. 2

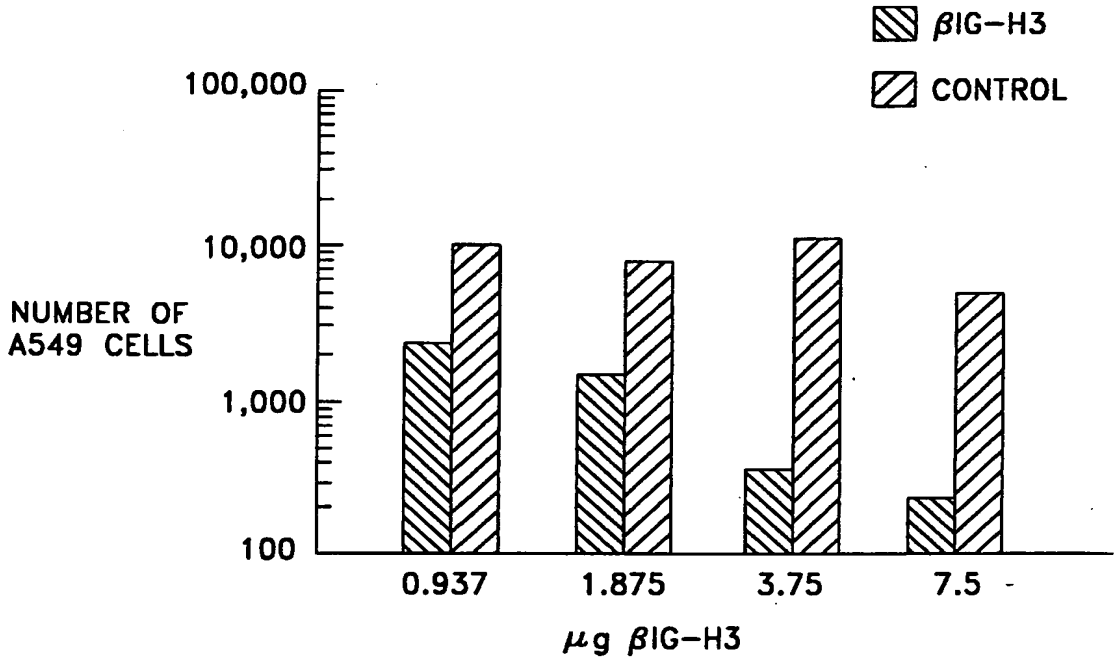

βIG-H3
CONTROL
100,000
10,000
1,000
100
NUMBER OF A549 CELLS
0.937
1.875
3.75
7.5
μg βIG-H3

FIG. 3

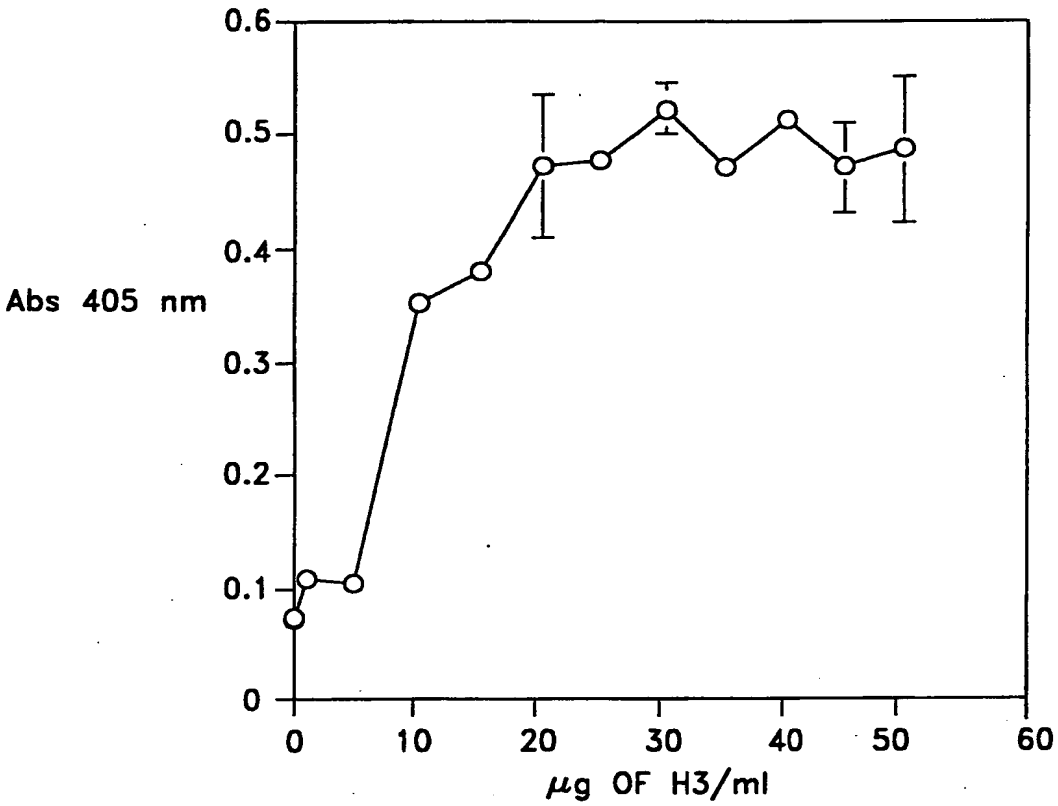
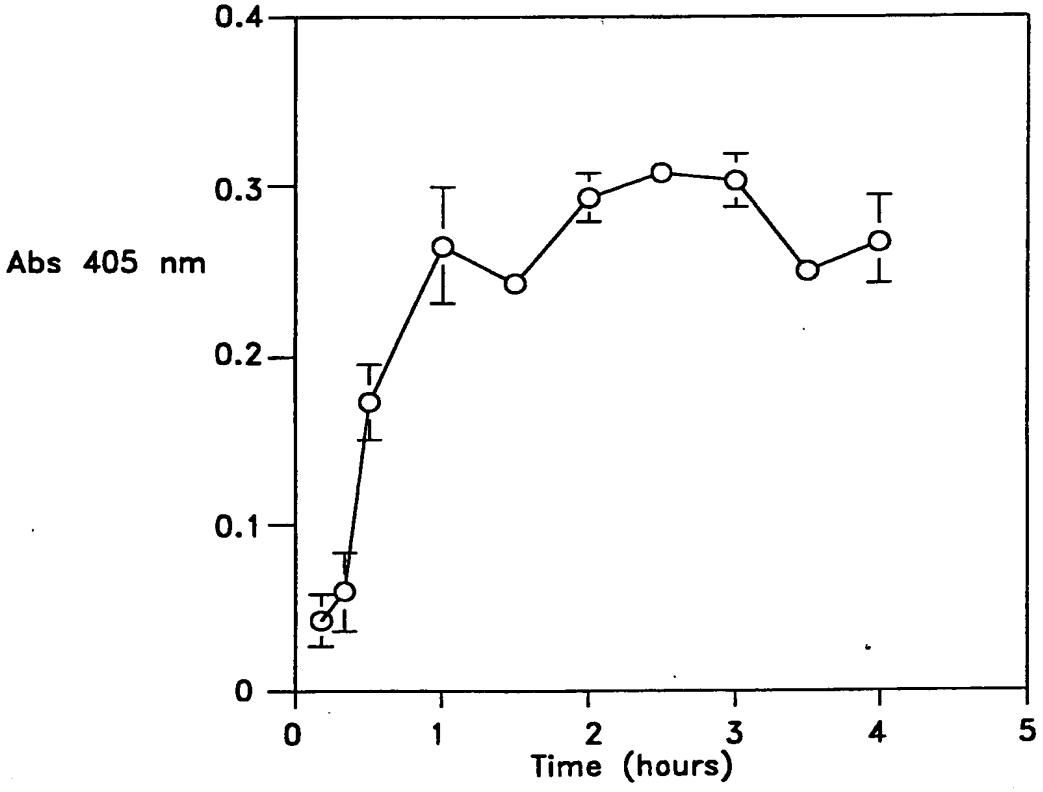


FIG. 4



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/08414

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :Please See Extra Sheet.
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/409, 410, 413, 418, 419, 443, 445, 462, 488, 85.6, 455, 46, 47; 435/178, 179, 180; 514/2, 8, 12; 530/350

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO, A, 92/14480 (AMGEN INC.) 03 September 1992, see entire document.	1-15, 18-32, 37-40
A	DNA AND CELL BIOLOGY, Volume 167, Number 7, issued 1992, Skonier et al, "cDNA Cloning and Sequence Analysis of βig-h3, a Novel Gene Induced in a Human Adenocarcinoma Cell Line after Treatment with Transforming Growth Factor-β", pages 511-522, see page 512, column 1, lines 43-60.	5, 14-15, 20-21
A	THE JOURNAL OF INVESTIGATIVE DERMATOLOGY, Volume 98, Number 4, issued April 1992, Chen et al, "Human Recombinant Transforming Growth Factor-β1 Modulation of Biochemical and Cellular Events in Healing of Ulcer Wounds", pages 428-435, see abstract and page 428, columns 1-2.	18-22, 28-32, 37, 39

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search
06 SEPTEMBER 1995

Date of mailing of the international search report
05 OCT 1995

Name and mailing address of the ISA/US
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Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/08414

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	SCIENCE, Volume 237, issued 11 September 1987, Mustoe et al, "Accelerated Healing of Incisional Wounds in Rats Induced by Transforming Growth Factor-β", pages 1333-1335, see page 1335, column 3, lines 20-35.	18-22, 28-32, 37, 39
A	PHARMACEUTICAL RESEARCH, Volume 11, Number 2, issued February 1994, Zioncheck et al, "Pharmacokinetics and Tissue Distribution of Recombinant Human Transforming Growth Factor Beta$_1$ After Topical and Intravenous Administration in Male Rats", pages 213-220, see page 213, column 2, lines 26-49.	18-22, 28-32, 37, 39

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/08414

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☒ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
1-15, 18-32, 37-40.

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/08414

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6):

A61K 9/06, 9/08, 9/10, 9/12, 9/107, 9/70, 38/16, 47/38; C12N 15/09, 15/12; C07K 14/475, 17/02, 17/08, 17/10, 17/12

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

424/409, 410, 413, 418, 419, 443, 445, 462, 488, 85.6, 455, 46, 47; 435/178, 179, 180; 514/2, 8, 12; 530/350

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

APS, CAS ONLINE, MEDLINE, BIOSIS, SCISEARCH, EMBASE
search terms: transforming growth factor-beta induced, beta-ig-H3, fibroblast, carrier, solid support, solid phase, attachment, wound, ulcer, burn, laceration, incision

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

I. Claims 1-15, 23-27, 38 and 40, drawn to a solid support and a method of use by coating the support with H3 protein.

II. Claims 16-17, and 36, drawn to a method of inhibiting tumor growth by contacting the tumor with a DNA construct operably encoding H3.

III. Claims 18-22, 28-32, 37, and 39, drawn to a method of accelerating wound healing by contacting the wound with H3.

IV. Claims 33-35 drawn to a method of producing recombinant H3 protein.

The inventions listed as Groups I-IV do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

The products/processes of Groups I-IV do not share the same or a corresponding special technical feature in that a solid support and a method of use by coating the support with H3 protein of Group I, a method of inhibiting tumor growth by contacting the tumor with a DNA construct operably encoding H3 of Group II, a method of accelerating wound healing by contacting the wound with H3 of Group III and a method of producing recombinant H3 protein of Group IV do not require each other for their practice and have separate functions, all of which constitute different special technical features which define the contribution of each invention. Groups II-IV are drawn to methods having different goals, method steps, and starting materials which do not share the same or a corresponding special technical feature which define the contribution of each invention. In addition, with respect to Groups II and III, the method of inhibiting tumor growth and the method of accelerating wound healing can be practiced with compounds other than H3, and with respect to Group IV, H3 can be produced by a different method such as chemical synthesis. Since these special technical features are not shared by each product/process, the inventions of Groups I-IV do not form a single inventive concept within the meaning of Rule 13.2.